

PHOSPHOLIPASE INHIBITORS FOR THE TREATMENT OF CANCER

FIELD OF THE INVENTION

INS A

5 The present invention relates generally to a method of treating disease conditions by the administration of an inhibitor of phospholipase activity. More particularly, the present invention contemplates a method for facilitating apoptosis of cancer cells or otherwise reducing or preventing growth of cancer cells by inhibiting phospholipase activity. Even more particularly, the present invention contemplates the use of inhibitors of
10 phospholipase A₂ enzymes in the treatment and prophylaxis of cancer. The present invention further provides biological compositions comprising an inhibitor of phospholipase A₂ alone or in combination with other agents in the treatment of cancer.

BACKGROUND OF THE INVENTION

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Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

The increasing sophistication of diagnostic and surgical techniques is greatly facilitating
20 the treatment of cancer. However, despite the improvements in the diagnosis and surgical treatment of cancer, the development of efficacious yet non-harmful anti-cancer agents has been slow.

Cancer is a most serious and debilitating disease condition facing both the human and
25 animal populations. The term "cancer" covers a range of malignant cell conditions and encompassing relatively minor conditions as well as serious and generally fatal conditions. For example, gastric cancer is a major contributor of cancer-related deaths throughout the world. According to the World Health Organisation (1), in 1993, gastric cancer was the fourth leading cause of cancer death in the United States and the second leading cause of
30 cancer death in Japan. Colorectal cancer is the second leading cause of cancer death in the

- 2 -

United States (2). However, despite a greater understanding of the genetic bases of this type of cancer, non-surgical treatment of colorectal cancer has not been overly successful.

Recent reports have provided evidence for a role for aspirin and other non-steroidal anti-5 inflammatory drugs (NSAIDs) in reducing cancer development (3-7). However, prolonged use of these compounds can lead to adverse gastrointestinal side-effects. One common target for NSAIDs is the enzyme cyclooxygenase. This enzyme exists in two isoforms, referred to herein as "COX1" and "COX2". Most NSAIDs do not discriminate between COX1 and COX2 (8-10). COX1 is constitutively expressed in a number of cells 10 (11) whereas COX2 is inducible by, for example, growth factors and cytokines (12, 13). It is apparent, therefore, that COX2 gene expression is elevated in inflammatory cells and sites of inflammation.

COX1 and COX2 play important roles in physiological processes such as prostaglandin 15 biosynthesis. The latter is important since excessive prostaglandin production is implicated and associated with proinflammatory eicosanoid, inhibition of production of immune regulatory lymphokines, inhibition of T- and B-cell proliferation, inhibition of cytotoxic activity of natural killer cells, induction of immunosuppression-facilitating molecules (e.g. TNF and IL-10) and reduced apoptosis of colon cancer cells.

20 Another important component of the regulatory pathway to prostaglandin biosynthesis is phospholipase and, in particular, phospholipase A₂ (hereinafter referred to as "PLA₂").

During prostaglandin biosynthesis, membrane phospholipids are metabolised by 25 phospholipases. Phospholipases are carboxylic acid esterases classified as phospholipase (PL) A₁, A₂, B and the phosphodiesterases, which are specific for lecithins. PLA₂ removes the unsaturated fatty acid at the C-2 of glycerol. The product of PLA₂ activity is arachidonic acid which is then catalytically converted to prostaglandin via the COX enzymes.

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The inventors have surprisingly discovered that phospholipase inhibitors which target PLA₂ are useful for modulating cancer growth and development

SUMMARY OF THE INVENTION

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Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

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Sequence Identity Numbers for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography.

The subject specification contains nucleotide and amino acid sequence information

15 prepared using the programme PatentIn Version 2.0, presented herein after the bibliography. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information

20 provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (eg. <400>1, <400>2, etc).

25 The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine 30 or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other

than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

One aspect of the present invention contemplates a method for controlling the growth 5 and/or development of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a phospholipase inhibitor or a functional derivative or homologue thereof.

More particularly, the present invention contemplates a method for controlling the growth 10 and/or development of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a phospholipase inhibitor or a functional derivative or homologue thereof which phospholipase inhibitor or derivative or homologue reduces the levels and/or activities of a phospholipase to an extent to reduce the growth and/or development of cancer cells.

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Another aspect of the present invention provides a method for reducing the volume of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a phospholipase inhibitor or a functional derivative or homologue thereof which phospholipase inhibitor or a derivative or homologue reduces 20 the levels and/or activities of a phospholipase to an extent to reduce the growth and/or development of cancer cells.

A further aspect of the present invention contemplates a method for controlling the growth and/or development of a cancer or the volume of a cancer in an animal or avian species 25 said method comprising administering to said animal or avian species an effective amount of a PLA₂ inhibitor or a functional derivative or homologue thereof which PLA₂ inhibitor or a derivative or homologue reduces the levels and/or activities of one or more types of PLA₂ to an extent to reduce the growth and/or development and/or volume of the cancer.

30 Yet a further aspect of the present invention contemplates a method for controlling the

- 5 -

growth and/or development of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a PLA₂ inhibitor having an amino acid sequence substantially as set forth in any one or more of SEQ ID NOS: 1 to 11 or SEQ ID Nos: 12 to 33 or an amino acid sequence having at least 5 60% identity to any one or more of SEQ ID NOS: 1 to 11 or 12 to 33 or a functional derivative or homologue thereof which PLA₂ inhibitor or derivative or homologue reduces the level or activity of secretory PLA₂.

Yet another further aspect of the present invention provides a biological composition 10 useful for the treatment and/or prophylaxis of cancer in a target animal or bird such as a human, primate, livestock animal or companion animal said composition comprising a PLA₂ inhibitor such as but not limited to the PLA₂ defined by any one of amino acids sequences set forth in SEQ ID NOS: 1-11 or 12 to 33 or a derivative, homologue, analogue or functional equivalent thereof.

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Another aspect provides an agent for use in treating or preventing cancer, said agent comprising a PLA₂ inhibitor or a functional derivative, homologue or analogue thereof.

Yet another aspect of the present invention contemplates the use of a PLA₂ inhibitor or 20 functional derivative, homologue or analogue in the manufacture of a medicament for the treatment or prophylaxis of cancer in an animal (e.g. human) or bird.

BRIEF DESCRIPTION OF THE FIGURES

25 Figure 1 is a diagrammatic representation of the interaction between extracellular, membrane associated and cytosolic factors in the production of prostaglandins.

Figure 2 is a graphical representation of the effects of NSI inhibitor on BGC-823 cancer growth in nude mice following subcutaneous administration.

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- 6 -

Figure 3 is a graphical representation of the effects of NSI inhibitor on BGC-823 cancer growth in nude mice following intraperitoneal administration.

Figure 4 is a graphical representation of the effects of NS398 inhibitor on BGC-823 cancer growth in nude mice following subcutaneous administration.

Figure 5 is graphical representation of the effects of NS398 inhibitor on BGC-823 cancer growth in nude mice following intraperitoneal administration.

10 Figure 6 is a graphical representation of the effects of NSI plus NS398 inhibitors on BGC-823 cancer growth in nude mice following subcutaneous administration.

Figure 7 is a graphical representation of the effects of NSI plus NS398 inhibitors on BGC-823 cancer growth in nude mice following intraperitoneal administration.

15 Figure 8 is a graphical representation of the growth of BGC-823 and SGC-7901 cancers in nude mice.

Figure 9 is a graphical representation of the effects of NSI plus NS398 inhibitors on BGC-20 823 cancer growth in nude mice following subcutaneous administration.

Figure 10 is a graphical representation of the combined effects of NSI plus NS398 inhibitors on BGC-823 cancer growth in nude mice following subcutaneous administration.

25 Figure 11 is a graphical representation of the effects of NSI and NS398 inhibitors on BGC-823 cancer growth in nude mice following intraperitoneal administration.

Figure 12 is a graphical representation of the combined effects of NSI plus NS398 inhibitors on BGC-823 cancer growth in nude mice following intraperitoneal 30 administration.

- 7 -

Figure 13A is a graphical representation showing the inhibition of non-snake venom PLA₂ by NSI, dilution group 1.

Figure 13B is a graphical representation showing the inhibition of non-snake venom PLA₂'s by NSI, dilution group 2.

Figure 14A is a graphical representation showing the inhibition of snake venom PLA₂ enzymes with NSI, Day 1.

10 **Figure 14B** is a graphical representation showing the inhibition of snake venom PLA₂ enzymes with NSI, Day 2.

Figure 15 is a graphical representation showing the inhibition of rhPLA₂ by NSI.

15 A summary of SEQ ID Nos used herein is given in Table 1.

- 8 -

TABLE 1
SUMMARY OF SEQ ID NOS:

SEQ ID NO	DESCRIPTION
1	Amino acid sequence of NS1 α -chain including leader sequence
5 2	Amino acid sequence of <i>Oxyuranus scutellatus</i> PLA ₂ inhibitor α -chain
3	Nucleotide and amino acid sequences of <i>Oxyuranus microlepidotus</i> PLA ₂ inhibitor α -chain
4-11	Amino acid sequence of tryptic peptides of NS1 β -chain
12	N-terminal amino acid sequence of α -chain isoform 1 of NAI
13	N-terminal amino acid sequence of α -chain isoform 2 of NAI
10 14-18	Enzymatic digest of mature α -chain of NAI
19	N-terminal amino acid sequence of β -chain of NAI
20-29	Enzymatic digest of mature β -chain of NAI
30-32	Amino acid sequence of mature α -chain determined from DNA sequence (minus leader sequence) of NAI
33	Amino acid sequence of mature β -chain of NAI
15 34	Nucleotide sequence encoding SEQ ID NO:30 (mature α -chain minus leader sequence)
35	Nucleotide sequence encoding SEQ ID NO:31 (mature α -chain minus leader sequence)
36	Nucleotide sequence encoding SEQ ID NO:32 (mature α -chain minus leader sequence)
37	Nucleotide sequence encoding SEQ ID NO:33 (mature β -chain minus leader sequence)
38-40	Amino acid sequence of leader sequence of α -chain of NAI determined from nucleotide sequence
20 41	Amino acid sequence of leader sequence of β -chain of NAI determined from nucleotide sequence
42	Nucleotide sequence of SEQ ID NO:38 (leader sequence of α -chain of NAI)
43	Nucleotide sequence of SEQ ID NO:39 (leader sequence of α -chain of NAI)

- 9 -

SEQ ID NO	DESCRIPTION
44	Nucleotide sequence of SEQ ID NO:40 (leader sequence of α -chain of NAI)
45	Nucleotide sequence of SEQ ID NO:41 (leader sequence from β -chain of NAI)

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, the inventors have determined that an inhibitor of PLA₂ and in particular secretory PLA₂ (sPLA₂) is effective in controlling the growth and development of cancer.

Accordingly, one aspect of the present invention contemplates a method for controlling the growth and/or development of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a phospholipase inhibitor or a functional derivative or homologue thereof.

More particularly, a method for controlling the growth and/or development of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a phospholipase inhibitor or a functional derivative or homologue thereof which phospholipase inhibitor or derivative or homologue reduces the levels and/or activities of a phospholipase to an extent to reduce the growth and/or development of cancer cells.

The present invention is particularly directed to the treatment and prophylaxis of cancers in animals such as humans, primates, livestock animals (e.g. sheep, goats, horses, cows, donkeys) laboratory test animals (e.g. mice, rats, guinea pigs, rabbits, hamsters), companion animals (e.g. dogs, cats) and captive wild animals. The present invention also extends, however, to avian species such as but not limited to poultry birds (e.g. chickens, geese, ducks, turkeys), game birds (e.g. pheasant, wild ducks, peacocks, emus, ostriches) and caged birds. The preferred targets for cancer therapy are animals such as humans, primates and laboratory test animals. More preferably, the target is human.

Reference to "controlling the growth and/or development" of cancer includes the induction of apoptosis and/or necrosis in cancer cells as well as reducing, inhibiting or otherwise retarding growth of cancer cells or the risk of cancer cell development. An analysis of the

effect on cancer cell growth may be conducted by any means but is conveniently determined by the "volume" of cancer cell material. The term "controlling the growth and/or development" of cancer includes, therefore, controlling the volume of a cancer as well as reducing, inhibiting or otherwise retarding the volume of a cancer.

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Assessment of cancer cell death or apoptosis may be made by any convenient means such as but not limited to macroscopic examination, microscopic examination, the determination of metaphase frequency, the determination of the proportion of cells in the S-phase, examination of cell lysis, determination of nuclear damage, an analysis of nuclear

10 fragmentation and/or a determination of the percentage of cells with subdiploid DNA.

Accordingly, another aspect of the present invention provides a method for reducing the volume of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a phospholipase inhibitor or a 15 functional derivative or homologue thereof which phospholipase inhibitor or a derivative or homologue reduces the levels and/or activities of a phospholipase to an extent to reduce the growth and/or development of cancer cells.

The term "cancer" is used in its broadest sense and includes benign and malignant 20 leukemias, sarcomas and carcinomas. The cancers contemplated by the present invention may be simple (i.e. composed of a single neoplastic cell type), mixed (i.e. composed of more than one neoplastic cell type) or compound (i.e. composed of more than one neoplastic cell type and derived from more than one germ layer). Examples of simple cancers encompassed by the present invention include tumours of mesenchymal origin 25 (e.g. tumours of connective tissue, endothelial tissue, blood cells, muscle cells) and tumours of epithelial origin. Particular cancers contemplated by the present invention include fibrosarcoma, myxosarcoma, Ewing's sarcoma, granulocytic leukemia, basal cell carcinoma, colon cancer, gastric cancer and a variety of skin cancers.

30 The preferred phospholipase inhibitors of the present invention are those which inhibit

- 12 -

PLA₂.

Even more preferably, the phospholipase inhibitor inhibits more than one type of PLA₂ molecule.

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PLA₂ enzymes comprise several sub-types, for example human Type I PLA₂ which is derived from human pancreas (14, 15) and human type II which is derived from human synovium, amongst others. Another PLA₂ enzyme is type V PLA₂ (16).

10 Accordingly, another aspect of the present invention contemplates a method for controlling the growth and/or development of a cancer or the volume of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a PLA₂ inhibitor or a functional derivative or homologue thereof which PLA₂ inhibitor or a derivative or homologue reduces the levels and/or activities of one or more 15 types of PLA₂ to an extent to reduce the growth and/or development and/or volume of the cancer.

Preferably, the PLA₂ inhibitor inhibits more than one type of PLA₂ molecule.

20 Preferably, the PLA₂ inhibitor is in isolated form and may be a proteinaceous molecule, lipid and/or polysaccharide or may be in another chemical form.

The term "isolated" means that the PLA₂ inhibitor of the present invention is provided in a form which is distinct from that which occurs in nature, preferably wherein one or more 25 contaminants have been removed. Accordingly, the isolated PLA₂ inhibitor may be used in partially-purified or substantially pure form, in which a substantial amount of contaminants have been removed and/or is in a sequencably pure or substantially homogeneous form.

30 The term "sequencably pure" means that the isolated PLA₂ inhibitor is provided in a form

which is sufficiently purified to facilitate amino acid sequence determination using procedures known to those skilled in the art.

The term "substantially homogeneous" means that the isolated PLA₂ is at least about 75% 5 free of contaminants, more preferably at least about 80% free of contaminants, including 90-100% purity.

The preferred phospholipase inhibitor in accordance with the present invention is one which is derivable from the serum or other bodily fluid of a venomous animal such as a 10 venomous insect or venomous snake, amongst others.

PLA₂ inhibitors useful in the practice of the present invention is from the Australian tiger snake *Notechis scutatus* or the Tasmanian tiger snake *Notechis ater*. The present invention extends, however, to PLA₂ inhibitors from the serum or bodily fluid from a range of other 15 venomous animals including a range of venomous snakes. The present invention extends to PLA₂ inhibitors identified following natural product screening from, for example, plants, microorganisms, river and sea beds and aquatic and antarctic environments.

Examples of insects, snakes and aquatic animals from which a PLA₂ inhibitor may be 20 isolated include arachnids (eg. spiders, scorpions, mites, etc) insects (eg. wasps, bees, ants, fleas, etc), reptiles (eg. snakes, lizards, etc), amphibians (eg. toads, frogs) or aquatic animals (eg: fish, cephalopods, box jellyfish, Portuguese man-of-war jellyfish, blue-ringed octopus, etc), amongst others.

25 Examples of snakes include snakes from the family Colubridae (colubrid snakes such as species of the genera *Heterodon*, *Natrix*, *Regina*, *Clonophis*, *Thamnophis*, *Lampropeltis*, *Opheodrys*, *Coluber*, *Masticophis*, *Drymobius*, *Salvadora*, *Phyllorhynchus*, *Elaphe*, *Hydrodynastes*, *Ptyas*, *Calamaria*, *Lycodon*, *Mehelya*, *Boaedon*, *Farancia*, *Fordonia*, *Erpeton*, amongst others), Elapidae (cobras such as species of the genera *Ophiophagus*, 30 *Naja*, *Oxyuranus*, *Pseudohaje*, *Walterinnesia*, *Aspidelaps*, *Boulengerina*, *Dendroaspis*,

- 14 -

Bungarais, Calliophis, Maticora, Micurus, Micruroides, Acanthophis, Notechis and Australaps, amongst others), Hydrophiidae (sea snakes such as species of the genera *Laticauda*, *Aipysurus*, *Hydrophis* and *Enhydrina*, amongst others), Viperidae (vipers, such as species of the genera *Vipera*, *Echis*, *Cerastes*, *Bitis*, *Atractaspis* and *Causus*, amongst 5 others) and Crotalidae (pit vipers such as species of the genera *Crotalis*, *Sistrurus*, *Bothrops*, *Trimeresurus*, *Lachesus* and *Agkistrodon*, amongst others).

Particularly preferred snakes include snakes from the family Viperidae, such as *Vipera* spp. and *Bitis* spp., in particular, *V. russelli*, *A. bilineatus* and *B. alternatus*; the family 10 Crotalidae, such as the moccasin snakes and vipers (*Agkistrodon* spp.) and the rattlesnakes (*Crotalus* spp.), in particular *Crotalus atrox*; or the family Elapidae, such as but not limited to King cobra (*Ophiohagus hannah*); True cobras (*Naja* spp); Asian or Indian cobra (*N. naja*); Egyptian cobra (*N. haje*); Spitting cobra (*N. nigricollis*); Black-lipped cobra (*N. malenoleuca*); Cape cobra (*N. nivea*); Gold's tree cobra (*Pseudohaje goldii*); 15 Desert black snakes (*Walterinnesia* spp); Shield-nose snakes (*Aspidelaps* spp); Water cobras or water snakes (*Boulengerina* spp); Black mamba (*Dendroaspis polylepis*); Mamba (*D. angusticeps*); Kraits snake (*Bungarus* spp); Oriental coral snakes (*Calliophis* spp); Long-glanded coral snakes (*Manicora* spp); American coral snakes (*Micurus* spp); Southern coral snake (*M. frontalis*); Eastern coral snake or Harlequin snake (*M. fulvius*); 20 Western coral snake (*Micruroides* spp); Arizona coral snake (*M. euryxanthus*); Death adder (*Acanthophis antarcticus*); Australian tiger snakes (*Notechis* spp e.g. *N. scutatus* or *N. ater*); and Australian copperhead (*Australaps* spp), amongst others.

The present inventors have determined that the *N. scutatus* and *N. ater* PLA₂ inhibitors 25 inhibit more than one type of PLA₂ and in particular secretory PLA₂. The PLA₂ inhibitor may be substantially homogenous or may be in a partially-purified form by, for example, fractionation using anion exchange chromatography or a dialysed form by, for example, cation exchange chromatography. The inventors have further provided sequencably pure *N. scutatus* and *N. ater* PLA₂ inhibitors.

- 15 -

A PLA₂ inhibitor is a molecule which reduces the activity of a phospholipase enzyme compared to the activity of the phospholipase enzyme in the absence of the inhibitor. The preferred PLA₂ inhibitor is a peptide, polypeptide or protein.

5 Accordingly, a PLA₂ inhibitor is a substance, such as a peptide, polypeptide and protein, which is capable of inhibiting phospholipase enzyme activity. The inhibitor may also be a polypeptide aggregate such as dimer or other multimer of a polypeptide, fusion polypeptide, peptide fragment or a homologue, analogue or derivative thereof which is capable of inhibiting the catalytic activity of a phospholipase enzyme, in particular a PLA₂,
10 enzyme and more preferably more than one type of PLA₂ enzyme.

Reference herein to a "PLA₂ inhibitor" includes reference to any peptide fragments or parts derived from a polypeptide, polypeptide aggregate or fusion polypeptide or homologue, analogue or derivative thereof, which, although they may have no inhibitory 15 activity may nevertheless be useful in modulating a PLA₂ inhibitor by, for example, competition.

Those skilled in the art will be aware that the amount of phospholipase inhibitor which is required to achieve inhibition may vary, depending upon the phospholipase enzyme being 20 inhibited, the presence of other substances which may interfere with phospholipase activity inhibitor activity, in particular substances derived from the source tissue. Accordingly, the present invention is not to be limited by the quantity or amount of phospholipase inhibitor required to achieve a particular degree of inhibition of enzyme activity.

25 In a preferred embodiment of the invention, the PLA₂ protein inhibitor described herein is capable of inhibiting at least 20%, more preferably at least about 50-70% and even more preferably at least about 80% of the PLA₂ activity present in a biological sample such as secretory PLA₂ in serum or tissue fluid.

- 16 -

In particular, the phospholipase inhibitor of the present invention exemplified herein [*N. scutatus* PLA₂ inhibitor (NSI) and *N. ater* PLA₂ inhibitor (NAI)] have been shown by the inventors to inhibit all groups of PLA₂ enzymes against which it has been tested. The molar ratio of NSI:PLA₂ and NAI:PLA₂ are each believed to be about 1:1. It has an IC₅₀ value of about 1.5 μ M for recombinant human non-pancreatic type-II PLA₂. Additionally, NSI and NAI form a stable complex with notexin (a purified PLA₂ enzyme) as judged by elution from a size exclusion column and also prevents radioiodinated notexin from binding to isolated rat brain synaptosomes.

10 In one embodiment of the invention, the PLA₂ inhibitor is derived from the serum of an animal such as a snake or other reptile, which produces a venom having toxic PLA₂ activity in humans or other animals.

15 Hereinafter, the term "derived from" shall be taken to refer to the origin of an integer or group of integers from a specified source, but not to the exclusion of other possible source or sources of said integer or group of integers.

In a particularly preferred embodiment of the invention, the PLA₂ inhibitor is derived from a snake.

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In a most particularly preferred embodiment, the present invention provides an isolated PLA₂ inhibitory protein derived from *Notechis scutatus* (NSI) or *Notechis ater* (NAI) which is capable of inhibiting more than one type of PLA₂ or is a functionally equivalent, homologue, analogue or derivative thereof of said inhibitor.

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The present invention extends to all isoforms of NSI and NAI.

The present invention extends further to a PLA₂ inhibitor molecule wherein said molecule is capable of binding to the active site of the PLA₂ enzyme.

30

In a particularly preferred embodiment, the PLA₂ inhibitor molecules according to this embodiment are capable of forming an interactive site with a phospholipase enzyme to inhibit the activity of the enzyme.

5 As used herein, the term "interactive site" shall be taken to refer to the primary, secondary or tertiary structure of a phospholipase inhibitor of the present invention which is in physical relation with a phospholipase enzyme wherein said physical relation is required for the inhibitory activity of said inhibitor, or at least contributed to the inhibitory activity of said inhibitor.

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In a more preferred embodiment, a molecule which is capable of forming an interactive site with a phospholipase enzyme mimics the 3-dimensional structure (i.e. tertiary structure) of the *N. scutatus* PLA₂ inhibitor (NSI) or *N. ater* PLA₂ inhibitor (NAI) and, as a consequence, is capable of reproducing the NSI:PLA₂ or NAI:PLA₂ inhibitory

15 interaction.

In this regard, whilst not being bound by any theory or mode of action, the mechanism of interaction between NSI or NAI and the PLA₂ enzyme at least appears to be unique compared to the mode of interaction of other PLA₂ inhibitors with the specific enzymes 20 which they inhibit, thereby accounting for the generality of NSI or NAI inhibitory activity. Those skilled in the art will be aware that once the structure of the interactive site between NSI or NAI and a PLA₂ enzyme is established by standard X-ray crystallographic procedures, it is possible to synthesize peptides or other molecules (mimotypes) which are capable of reproducing the inhibitory function of NSI or NAI. Such mimotypes, whilst 25 capable of forming an interactive site with a phospholipase enzyme may not comprise the same amino acid sequence (i.e. primary structure) as the NSI or NAI α -chain and/or β -chain polypeptide(s). Furthermore, those skilled in the art will be aware that mimotypes may also comprise synthetic molecules such as chemical compounds or anti-idiotypic antibodies of the phospholipase inhibitor of the invention capable of forming an 30 interactive site with a phospholipase. Those skilled in the art will also be aware that

mimotypes may be presented on a carrier molecule or embedded therein, such that the mimotype moiety is presented in a functional conformation capable of inhibiting phospholipase enzyme activity. Accordingly, the present invention clearly extends to any molecule or composition of matter which at least comprises a mimotype of NSI or NAI or 5 the interactive site thereof.

Carrier molecules for presenting a mimotype may comprise amino acid sequences presented as an in-frame fusion polypeptide with a polypeptide mimotype or alternatively, associated with a polypeptide mimotype by means of a disulfide bridge or other covalent 10 bond formation, van der Waals interaction or ionic interaction, amongst others. Alternatively, wherein the mimotype moiety is a chemical compound, the mimotype may be embedded into a polypeptide carrier by any means known to those skilled in the art.

Carrier molecules for presenting a mimotype may also comprise polysaccharide molecules, 15 nucleic acid molecules such as RNA or DNA, biologically inert carriers such as tungsten or gold, amongst others, polymers such as starches, dextrans, glycogen, Percoll (Trademark of Pharmacia Fine Chemicals) or Ficoll (Trademark of Pharmacia Fine Chemicals), amongst others, agarose, polyacrylamide or other couriers known to those in the pharmaceutical and/or biomolecular engineering industries.

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Another aspect of the present invention provides an isolated phospholipase inhibitory protein which at least comprises an amino acid sequence which is at least about 40% identical to SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or is a homologue, analogue or derivative thereof. The amino acid sequences set forth in SEQ ID NOS: 4-11 relate to 25 tryptic peptides of the *N. scutatus* PLA₂ inhibitory protein β -chain. The amino acid sequence set forth in SEQ ID NO: 1 relates to the derived amino acid sequence of the *N. scutatus* PLA₂ inhibitory protein α -chain. The amino acid sequence set forth in SEQ ID NO: 1 comprises the complete NSI α -chain polypeptide, including a 19 amino acid N-terminal leader peptide which is absent from the N-terminus of the mature protein. The 30 amino acid sequence set forth in SEQ ID NO: 2 relates to the derived amino acid sequence

of the *Oxyuranus scutellatus* PLA₂ inhibitory protein α -chain. The amino acid sequence set forth in SEQ ID NO: 3 relates to the derived amino acid sequence of the *Oxyuranus microlepidotus* PLA₂ inhibitory protein α -chain.

5 Preferably, the percentage identity is at least about 50%, more preferably at least about 60% and even more preferably at least about 75% identical to the NSI α -chain polypeptide set forth in SEQ ID NO: 1 or the *Oxyuranus spp.* polypeptides set forth in SEQ ID Nos: 2 or 3, still more preferably, the percentage identity is at least about 85%, and even more preferably at least about 95% identical to SEQ ID NO: 1 or 2 or 3.

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The percentage identity to the β -chain polypeptide is preferably at least about 40% identical to any one of SEQ ID NOS: 4 to 11 and more preferably at least about 50%, even more preferably at least about 80% and still more preferably at least about 95% identical thereto.

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Yet another aspect of the present invention provides an isolated phospholipase inhibitory protein which comprises the amino acid sequence substantially as set forth in any one or more of SEQ ID Nos 12 to 33 or a sequence having at least 40% identity thereto or an amino acid sequence encoded by a nucleotide sequence substantially as set forth in one or 20 more of SEQ ID Nos 34 to 37 or a nucleotide sequence having at least 40% identity thereto or capable of hybridizing to any one of SEQ ID Nos 34 to 37 under low stringency conditions at 42°C. The amino acid and nucleotide sequences set forth in SEQ ID NOS: 12-45 are summarized in Table 1.

25 Reference herein to a low stringency at 42°C includes and encompasses from at least about 1% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or

high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41 (G+C)\%$ [19]. However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (20).

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, 10 "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, 15 nucleotide and sequence comparisons are made at the level of identity rather than similarity. Any number of programs are available to compare nucleotide and amino acid sequences. Preferred programs have regard to an appropriate alignment. One such program is Gap which considers all possible alignment and gap positions and creates an alignment with the largest number of matched bases and the fewest gaps. Gap uses the 20 alignment method of Needleman and Wunsch (19). Gap reads a scoring matrix that contains values for every possible GCG symbol match. GAP is available on ANGIS (Australian National Genomic Information Service) at website <http://mell.angis.org.au..>

25 The present invention clearly extends to the use of the full-length amino acid sequences of both the precursor and mature α -chain and β -chain of the *N. scutatus* PLA₂ inhibitor or *N. ater* PLA₂ inhibitor and high molecular weight and to heteropolymers and recombinant and isolated forms thereof, including fusion polypeptides.

30 In the present context, "homologues" of a phospholipase inhibitory protein or PLA₂ inhibitory

protein refer to those polypeptides, enzymes or proteins which have a similar inhibitory activity to the NSI or NAI and are at least about 40% identical thereto, notwithstanding any amino acid substitutions, additions or deletions. Homologues may comprise fusion polypeptides between α -chain and β -chain polypeptides with or without additional

5 "spacer" sequences there between to facilitate folding and the ability of said fusion polypeptide to form an interactive site with a phospholipase enzyme. A homologue may be isolated or derived from the same species as the particular PLA₂ inhibitory protein exemplified herein (e.g. *N. scutatus* or *N. ater*) or alternatively, from a different species or a mixture of same.

10

Furthermore, the amino acids of a homologous polypeptide may be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, charge or antigenicity, and so on.

15 "Analogues" encompass PLA₂ inhibitors and polypeptides which are at least about 40% identical to the NSI or NAI or the interactive site thereof, notwithstanding the occurrence of any non-naturally occurring amino acid analogues therein. "Analogues" also encompass polypeptide mimotypes of the phospholipase inhibitor herein described.

20 The term "derivative" in relation to a PLA₂ inhibitor shall be taken to refer hereinafter to mutants, parts or fragments derived from the functional NSI or NAI or homologues or derivatives thereof which may or may not possess the inhibitory activity of the functional protein. Derivatives include modified peptides in which ligands are attached to one or more of the amino acid residues contained therein, such as carbohydrates, enzymes,

25 proteins, polypeptides or reporter molecules such as radionuclides or fluorescent compounds. Glycosylated, fluorescent, acylated or alkylated forms of the subject peptides are particularly contemplated by the present invention. Additionally, derivatives of a PLA₂ inhibitory protein which comprise fragments or parts of an amino acid sequence disclosed herein are within the scope of the invention, as are homopolymers or

30 heteropolymers comprising two or more copies of the subject polypeptides. Procedures for

derivatizing peptides are well-known in the art.

Particularly preferred analogues and derivatives of the NSI or NAI polypeptides exemplified herein comprise an amino acid sequence which is capable of binding to the 5 active site of a phospholipase enzyme and/or capable of forming an interactive site with a phospholipase enzyme.

Substitutions which may be included in a homologue, analogue or derivative of any one of SEQ ID NOS: 1 to 3 and/or 4 to 11 and/or 12 to 33 or a phospholipase inhibitor 10 polypeptide comprising amino acid alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as "conservative", in which case an amino acid residue contained in a phospholipase inhibitory protein is replaced with another naturally-occurring amino acid of similar character, for example $\text{Gly} \leftrightarrow \text{Ala}$, $\text{Val} \leftrightarrow \text{Ile} \leftrightarrow \text{Leu}$, $\text{Asp} \leftrightarrow \text{Glu}$, $\text{Lys} \leftrightarrow \text{Arg}$, 15 $\text{Asn} \leftrightarrow \text{Gln}$ or $\text{Phe} \leftrightarrow \text{Trp} \leftrightarrow \text{Tyr}$.

Substitutions encompassed by the present invention may also be "non-conservative", in which an amino acid residue which is present in a phospholipase inhibitory protein is substituted with an amino acid having different properties, such as a naturally-occurring 20 amino acid from a different group (eg. substituted a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-conventional amino acid.

Amino acid substitutions are typically of single residues, but may be of multiple residues, 25 either clustered or dispersed.

Naturally-occurring amino acids include those listed in Table 2A. Non-conventional amino acids encompassed by the invention include, but are not limited to those listed in Table 2B.

Amino acid deletions will usually be of the order of about 1-10 amino acid residues, while insertions may be of any length. Deletions and insertions may be made to the N-terminus, the C-terminus or be internal deletions or insertions. Generally, insertions within the amino acid sequence will be smaller than amino- or carboxyl-terminal fusions and of the 5 order of 1-4 amino acid residues.

Preferably, the phospholipase inhibitory protein of the invention or a homologue thereof comprises polypeptide chains having an estimated molecular weight of about 25 kDa or 30 kDa as determined by SDS/PAGE or alternatively, about 22-23 kDa or 19-20 kDa as 10 determined by mass spectrometry or alternatively, a fusion polypeptide comprising said polypeptide chains.

Wherein the phospholipase inhibitory protein is a multimeric protein, such as a heteropolymer of α -chain and β -chain polypeptides, it is also preferred that it exist as a 15 trimeric protein having a molecular weight in the range of about 76 kDa to about 120 kDa, more preferably about 84 kDa to about 110 kDa.

In a particularly preferred embodiment of the invention, the phospholipase inhibitory protein or a homologue or analogue thereof is a heterotrimeric $\alpha_2:\beta_1$ protein having an 20 estimated molecular weight of about 110 kDa.

The present invention clearly extends to fusion polypeptides comprising one or more α -chain and β -chain polypeptides and mimotypes thereof.

25 The range provided herein for the estimated molecular weight of a PLA₂ inhibitory protein is merely an approximation and some variation in this estimate may occur. Those skilled in the art will be aware that some variation in the estimated molecular weight of a polypeptide may occur, depending upon the conditions employed to determine said molecular weight.

- 24 -

TABLE 2A

Amino Acid	Three-letter Abbreviation	One-letter Symbol
5		
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
10 Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
15 Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
20 Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
25 Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

- 25 -

TABLE 2B

Non-conventional amino acid	Code	Non-conventional amino acid	Code
5 α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
α -amino- α -methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasu
		L-N-methylaspartic acid	Nmasp
10 aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl- carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
15 D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
20 D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
25 D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
30 D-threonine	Dthr	L-norleucine	Nle

D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
D-valine	Dval	α -methyl- γ -aminobutyrate	Mgabu
D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
5 D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
10 D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
D- α -methylleucine	Dmleu	α -naphthylalanine	Anap
D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
15 D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
20 D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D- α -methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D- α -methylvaline	Dmval	N-cyclododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
25 D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
30 D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)	

- 27 -

		glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))
5		glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylethyl)
		glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine
10	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine
	N-methylglycine	Nala	D-N-methylphenylalanine
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine
	N-(2-methylpropyl)glycine	Nieu	D-N-methylthreonine
15	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine
	D-N-methyltyrosine	Dnmtyr	N-methyl- α -naphthylalanine
	D-N-methylvaline	Dnmval	N-methylpenicillamine
	γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine
	L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine
20	L-ethylglycine	Etg	penicillamine
	L-homophenylalanine	Hphe	L- α -methylalanine
	L- α -methylarginine	Marg	L- α -methylasparagine
	L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine
	L- α -methylcysteine	Mcys	L-methylethylglycine
25	L- α -methylglutamine	Mgln	L- α -methylglutamate
	L- α -methylhistidine	Mhis	L- α -methylhomo
			phenylalanine
	L- α -methylisoleucine	Mile	N-(2-methylthioethyl)
			glycine
30	L- α -methylleucine	Mleu	N- α -methyllysine
			Mlys

L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
L- α -methylserine	Mser	L- α -methylthreonine	Mthr
5 L- α -methyltryptophan	Mtrp	L- α -methyltyrosine	Mtyr
L- α -methylvaline	Mval	L-N-methylhomo phenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
10 1-carboxy-1-(2,2-diphenyl- ethylamino)cyclopropane	Nmbc		

Reference to chemical analogues also includes reference to chemically synthesised
 15 molecules or molecules identified following screening of chemical libraries as well as
 molecules detected following, for example, natural product screening. Useful sources for
 screening for natural products include coral reefs and sea beds, plants, microorganisms
 and aquatic and antarctic environments.

20 The PLA₂ inhibitor or homologue, analogue or derivative thereof herein described is
 useful in the prophylaxis and treatment of cancer.

Although not intending to limit the present invention to any one theory or mode of action,
 it is proposed that phospholipase inhibitors alter the regulatory pathway associated with
 25 prostaglandin production. After analysing the literature, the inventors summarized
 diagrammatically the regulation of prostaglandin synthesis. This is shown in Figure 1.
 This figure shows the interaction between extracellular, membrane associated and
 cytosolic factors in the production of prostaglandin.

30 Importantly, Figure 1 shows that secretory PLA₂ (sPLA₂) is capable of down-regulating

expression or otherwise reducing the activity of the cyclooxygenase, COX2.

Although not wishing to limit the present invention to any one theory or mode of action, it is proposed herein that secretory PLA₂ has a regulatory effect on a cyclooxygenase and in 5 particular COX2.

In accordance with the present invention, it is proposed that the administration of a PLA₂ inhibitor such as NSI or NAI or an aforementioned equivalent, derivative or homologue thereof inhibits secretory PLA₂, which thereby reduces expression of COX2. This in turn 10 reduces the catalytic conversion of arachidonic acid to prostaglandin.

Accordingly, another aspect of the present invention contemplates a method for controlling the growth and/or development of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a PLA₂ 15 inhibitor having an amino acid sequence substantially as set forth in any one or more of SEQ ID NOs: 1 to 11 or an amino acid sequence having at least 60% identity to any one or more of SEQ ID NOs: 1 to 11 or a functional derivative or homologue thereof which PLA₂ inhibitor or derivative or homologue reduces the level or activity of secretory PLA₂.

20 In a particular embodiment the present invention contemplates a method for controlling the growth and/or development of a cancer in an animal or avian species said method comprising administering to said animal or avian species an effective amount of a PLA₂ inhibitor having an amino acid sequence substantially as set forth in any one or more of SEQ ID NOs: 1 to 11 or 12 to 33 or an amino acid sequence having at least 60% identity 25 to any one or more of SEQ ID NOs: 1 to 11 or 12 to 33 or a functional derivative or homologue thereof which PLA₂ inhibitor or derivative or homologue reduces the level or activity of secretory PLA₂, thereby reducing expression of a genetic sequence encoding a cyclooxygenase or reducing cyclooxygenase activity.

30 Yet another aspect of the present invention provides a biological composition useful for the

treatment and/or prophylaxis of cancer in a target animal or bird such as a human, primate, livestock animal or companion animal said composition comprising a PLA₂ inhibitor such as but not limited to the PLA₂ defined by any one of amino acids sequences set forth in SEQ ID NOs: 1-11 or 12 to 33 or a derivative, homologue, analogue or 5 functional equivalent thereof.

The biological composition according to this aspect of the present invention may also contain other active molecules such as anti-cancer agents, immune-potentiating molecules and/or pharmaceutical compounds which diminish any side-effects of the PLA₂ inhibitors 10 or other active molecules.

The active molecule(s) of the biological composition is/are contemplated to exhibit PLA₂ inhibitory activity and consequently anti-cancer activity in animals and birds when administered by any means including by intravenous, intraperitoneal, sub-cutaneous, topical 15 or oral administration. Variations in dosage administration occur depending, for example, on the activity of the phospholipase enzyme required to be inhibited and the IC50 of the inhibitor, the intended purpose of administration, such as whether for use as an anti-inflammatory agent or as an anti-toxin and particularly in the case of toxic poisoning and the delay between the onset of symptoms and the commencement of treatment. Dosage regimen 20 may be adjusted without undue experimentation by those skilled in the art to provide the optimum therapeutic response. For example, several divided doses may be administered in one or more of daily, hourly, weekly or monthly or in other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

25 The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or 30 both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, sachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid. The active ingredient may also be presented as a bolus, electuary or 5 paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with 10 a binder (e.g. inert diluent, preservative disintegrant (e.g. sodium starch glycolate, cross-linked polyvinyl pyrrolidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

15 Tablets or powders or granules may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Additionally, sweeteners or dietary formulae may be included to improve their palatability to a specific animal subject. Optionally, such solid compositions be provided with an enteric 20 coating, to provide release in parts of the gut other than the stomach.

The active compounds may also be administered in dispersions prepared in glycerol, liquid polyethylene glycols, and/or mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of 25 microorganisms.

Biological compositions suitable for parenteral administration include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile 30 and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating

action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating 5 such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the 10 injectable compositions can be brought about, for example, by the use in the compositions of agents delaying absorption.

Sterile injectable solutions are prepared by incorporating the active molecules in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as 15 required, followed by filter sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active molecule(s) into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which 20 yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

The biological compositions of the present invention may also be delivered by a live delivery system such as using a bacterial expression system to express the PLA₂ inhibitory protein in 25 bacteria which can be incorporated into gut flora. Alternatively, a viral expression system can be employed. In this regard, one form of viral expression is the administration of a live vector generally by spray, feed or water where an infecting effective amount of the live vector (e.g. virus or bacterium) is provided to the animal. Another form of viral expression system is a non-replicating virus vector which is capable of infecting a cell but not 30 replicating therein. The non-replicating viral vector provides a means of introducing to the human or animal subject genetic material for transient expression therein to produce the

PLA₂ inhibitory protein. The mode of administering such a vector is the same as a live viral vector.

The carriers, excipients and/or diluents utilised in the biological compositions of the present 5 invention should be acceptable for human or veterinary applications. Such carriers, excipients and/or diluents are well-known to those skilled in the art. Carriers and/or diluents suitable for veterinary use include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Except insofar as any conventional media or agent are incompatible with the active 10 ingredient, use thereof in the composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The compositions of this invention may include other agents conventional in the art. For example, compositions suitable for oral administration may include such further agents as 15 dietary formulae, binders, sweeteners, thickeners, flavouring agents disintegrating agents, coating agents, preservatives, lubricants and/or time delay agents. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose, polyvinylpyrrolidone, xanthan gum, bentonite, alginic acid or agar. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, 20 orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

25

The present invention further provides an agent for use in treating or preventing cancer, said agent comprising a PLA₂ inhibitor or a functional derivative, homologue or analogue thereof.

30 Still another aspect of the present invention contemplates the use of a PLA₂ inhibitor or functional derivative, homologue or analogue in the manufacture of a medicament for the

- 34 -

treatment or prophylaxis of cancer in an animal (e.g. human or bird).

The present invention is further described by the following non-limiting Examples.

- 35 -

EXAMPLE 1

Purification of Phospholipase A₂ Inhibitor from Snake Blood

Tiger snake (*N. scutatus*) and Tasmanian tiger snake (*N. ater*) blood were collected and 5 allowed to clot. The blood is then centrifuged at 1,500 x g for 15 minutes. The serum is then collected and stored at -20°C. Serum was extensively dialysed against 0.01M ammonium acetate (NH₄OAc), pH 7.0. The *N. scutatus* phospholipase A₂ inhibitor (NSI) and *N. ater* (NAI) were purified using anion exchange chromatography.

10 Dialysed serum was loaded (up to 15mL at -20mg/mL) onto a DEAE-Sephacel column (20 x 1.5cm) that has been equilibrated with 0.01M NH₄OAc, pH 7.0 at a flow rate of 0.5mL/min. A step gradient was then developed as follows; 0.1 NH₄OAc, 0.25M NH₄OAc, 0.5 NH₄OAc and 1.0M NH₄OAc (all pH 7.0). The eluent was monitored at 280nm with an Isco type 11 detector. The concentration of NH₄OAc was not increased 15 until the preceding peak has fully eluted. NSI and NAI were eluted the 0.5M NH₄OAc step. The procedure was performed at 4°C.

The sample was then concentrated by lyophilisation and then resuspended in water and stored at -20°C. Alternatively, if a large volume was collected (>15mL), the sample was 20 concentrated using an Amicon ultrafiltration device fitted with a YM 10 membrane. This semi-purified preparation (SPP) of NSI or NAI was approximately 90-95% pure.

NSI and NAI can be purified to >98% purity using cation exchange chromatography. A Mono-S HR 5/5 column was equilibrated with 10mM sodium acetate pH 5.5. The SPP 25 NSI or NAI fraction was applied and a gradient developed with 430mM sodium acetate pH 5.5 as follows:

- (i) 0-3 minutes 0%;
- (ii) 3-8 minutes 0-20%;
- (iii) 8-20 minutes 20-40%;
- 30 (iv) 20-25 minutes 40-60%; and

- 36 -

(v) 25-30 minutes 60-100%.

NSI and NAI eluted in the 20-40% section of the gradient. (Figure 1a and 1b).

The amino acid sequence for NAI are shown in SEQ ID NOS 12 to 33. Corresponding 5 nucleotide sequences are shown in SEQ ID NOS 34 to 37. The amino acid sequence of the leader system and corresponding nucleotide sequence are shown in SEQ ID NOS 38 to 45.

EXAMPLE 2

10 Phospholipase A₂ assays and inhibition of Phospholipase A₂ activity by NSI

Phospholipase A₂ activity was assigned using a modification of the method of Radvanyi *et al.* (17). This assay is based on the ability to measure the fluorescence emitted by an artificial substrate after it has been cleaved by a PLA₂ enzyme. The level of fluorescence 15 is proportional to the amount of cleaved substrate which is in turn proportional to enzymatic activity. The phospholipid substrate, labelled in the sn-2 position with 10-pyrenyldecanoic acid, forms micelles upon addition to the reaction medium. The fluorescence of the substrate is quenched by pyrene-pyrene interactions. Upon hydrolysis the free 10-pyrenyldecanoic acids are absorbed by bovine serum albumin (BSA) and the 20 fluorescence emitted is measured. The artificial substrate 1-hexadecanoyl-2-(1-predecanoyl)-sn-glycero-3-phosphocholine (10pPC [Molecular Probes, Inc.]) was dissolved (1mg) in 5.87mL 95% v/v ethanol to yield a 0.2M stock solution. 200 μ L aliquots were stored at -20 C for up to 3 months.

25 To 1mL of assay buffer (50mM Tris [hydroxymethyl]methylamine-HCl[Tris]), pH7.5, 100mM NaCl, and 1mM ethylenediaminetetra-acetic acid [EDTA]) the following were added sequentially; 16 μ L of a 1:0.6 v/v mixture of 10% w/v BSA and 1M CaCl₂ (0.1% and 2 μ M final concentration respectively), 10 μ L 10pPC stock solution, injected quickly to facilitate micellar formation. To this, 35 μ L of a test sample, PLA₂ source 30 plus SPP or water, or saline/BSA, was added. This solution was mixed well with shaking.

The substrate was excited at 345nm and the fluorescent spectrometer for 4 minutes.

EXAMPLE 3

Inhibition of non-snake venom phospholipase A₂ enzymes by

5 *N.scutatus* phospholipase A₂ inhibitor

Phospholipase A₂ enzyme activity assays were performed as described in Example 2. The assay was performed as above except that 10pPG (1-hexadecanoyl-2-(1-palmitoyl)-sn-glycero-3-phosphoglycerol, ammonium salt) was used as the substrate, because most of the 10 non-snake venom PLA₂s are not active on 10pPC. Also, saline, rather than water was used for the negative control.

PLA₂ enzymes were diluted to achieve an enzyme activity sufficient to produce a change of 250 fluorescent units over 70-80 seconds in the enzyme assay, in the absence of 15 inhibitor. Samples tested were; *N. scutatus* venom (positive control), bee venom phospholipase A₂ (*Apis mellifera*), porcine pancreatic phospholipase A₂ PLA₂ (*Sus scrofa*), and osteo-arthritis synovial fluid aspirates and rheumatoid arthritis-synovial fluid aspirates. Dilutions of phospholipase A₂-containing samples which were used were as follows; *N.scutatus* venom 1/30, bee venom phospholipase A₂ 1/400, porcine 20 pancreatic phospholipase A₂ 1/3, all 1mg/ml. Osteo-arthritis, undiluted to 1/10 and rheumatoid-arthritis-synovial, 1/30, 25-36mg/mL total protein. It should be noted that not all of the OA or RA samples meet with the activity criteria of 250 fluorescent intensity units over 70-80 seconds, however, the activity was consistent and measurable.

25 Dilutions of the SPP varied according to the phospholipase A₂ tested. Two dilution groups were used for a 7.13mg/mL solution of the SPP:

Group 1; 1/14, 1/50, 1/330 and 1/660. Phospholipase A₂ sources challenged with this group were *N.scutatus* venom, porcine pancreatic phospholipase A₂ and bee venom 30 phospholipase A₂.

Group 2; 1/2, 1/7, 1/14 and 1/50. Phospholipase A₂ sources challenged with this group were, all OA and RA samples.

As shown in Figure 13A, the SPP fraction of *N. scutatus* phospholipase A₂ inhibitor 5 strongly inhibited bee venom phospholipase A₂ at all concentrates tested. A 50% inhibition of porcine pancreas phospholipase A₂ was observed at a 1/4 dilution of SPP.

As shown in Figure 13B, the SPP fraction of *N. scutatus* phospholipase A₂ inhibitor significantly inhibited the three osteoarthritis samples tested, with about 40-60% inhibition 10 of enzyme activity being observed at a 1/2 dilution of SPP. In two of the three samples tested, about 50% inhibition of phospholipase A₂ activity was observed at the 1/7 dilution level of SPP. Weak, albeit detectable inhibition of phospholipase A₂ in the rheumatoid arthritis sample tested was also detected at the 1/4 dilution of SPP.

15 These data indicate that the *N. scutatus* venom phospholipase A₂ inhibitor is a broad-spectrum inhibitor of non-snake venom-derived phospholipase A₂ activities.

EXAMPLE 4

20 **Inhibition of a variety of snake venom phospholipase A₂ activities
by partially-purified *N. scutatus* phospholipase A₂ inhibitor**

Using the SPP fraction prepared according to Example 1, inhibition of the phospholipase A₂ activities of a wide range of snake venoms was tested. The venoms tested were; *N. scutatus* (homologous venom), *P. textilis*, *N. melanoleuca* (family; Elapidae), *V. russelli* 25 (family; Viperidae), *A. bilineatus*, *B. alternatus* and *C. atrox* (family; Viperidae, subfamily; Crotalinae).

First, an appropriate dilution of venom was established for use in the assay described in Example 2. The criteria required a substantial change in fluorescent intensity over a 30 relatively short period of time. Venoms were diluted to achieve a phospholipase A₂

enzyme activity sufficient to produce a change of 250 fluorescent intensity units over 70-80 seconds in the absence of any inhibitor. As such all venoms showed similar PLA₂ activity in the assay. A 1mg/mL solution of each venom was made up fresh when it was to be tested. Dilutions (of the 1mg/mL solution) used in the assay are as follows;

5 *N.scutatus* 1/200, *P.textilis* 1/20, *N.melanoleuca* 1/150, *V.russelli* 1/15, *A.bilineatus* 1/20, *B.alternatus* 1/10 and *C.atrox* 1/10.

The SPP fraction was also diluted prior to testing against each venom. The dilutions were; 1/2, 1/8, 1/12, 1/50, 1/100 and 1/200 of a 1.11mg/mL solution.

10

The SPP dilutions were incubated with each diluted venom sample in the ratio 2.5:1 (v/v) before assaying phospholipase A₂ enzyme activity. Three assays were performed for each dilution of SPP on each day. Control samples were assayed both before and after each dilution was tested. The control consisted of venom plus water in the same ratio as the 15 SPP:venom. Three batches were assayed daily with separate controls for each batch. All samples were prepared at the same time and then selected randomly for testing. All samples being tested were kept on ice. Samples not used immediately were stored at -20°C.

20 Results were determined as percentage inhibition compared to control values (Figures 14A and 14B). The SPP fraction of *N.scutatus* phospholipase A₂ inhibitor was most effective at inhibiting the activities of *N.scutatus* snake venom phospholipase A₂, with at least 80% inhibition of the related *N.melanoleuca* phospholipase A₂ being observed at all dilutions of SPP tested. Significant inhibition of phospholipase A₂ activities derived from 25 the more distantly related species were also observed at high concentrations of the SPP fraction, wherein 50% inhibition of *V.russelli* phospholipase A₂ was observed at a 1/25 dilution of SPP and a 50% inhibition of the *A.bilineatus* and *B.alternatus* phospholipase A₂ activities was observed at about a 1/12 dilution of SPP and a 50% inhibition of the *P.textilis* and *C.atrox* phospholipase A₂ activities was observed at about a 1/2-1/8 dilution 30 of SPP.

- 40 -

These data indicate that the *N. scutatus* venom phospholipase A₂ inhibitor is a broad-spectrum inhibitor of snake venom phospholipase A₂ enzymes.

5

EXAMPLE 5

Mixed micelle assay of recombinant human type II phospholipase A₂ and inhibition of enzyme activity using *N. scutatus* phospholipase A₂ inhibitor

An alternative assay of phospholipase A₂ activity was a mixed 10 micelle phosphatidylethanolamine (PE/sodium deoxycholate (DOC) assay modified from a method of Seilhamer *et al* (18). This assay is particularly suited to quantifying recombinant human phospholipase A₂ activity as it utilises a PE/DOC substrate. The PE substrate was prepared by dissolving freshly desiccated [¹⁴C]PE (Amersham) in 2% DOC, then diluting this to 0.22 μ moles PE and 0.04% DOC per sample in assay buffer (50mM 15 Tris-HCl, pH 8.5, 2mM CaCl₂, 150mM NaCl, 0.04% DOC). The sample was prepared by mixing 10 μ L of the test material with 10 μ L 10mM Tris-HCl, pH 7.4 and incubating for 10 minutes at 37°C. The reaction was started by the addition of 25 μ L pre-warmed substrate and terminated by the addition of 10 μ L 100mM EDTA. The reaction mixture (30 μ L) was spotted and dried onto silica TLC plates. The plates were chromatographed 20 using chloroform:methanol:acetic acid (90:10:1) as solvent. The dried plates were then exposed overnight with Kodak X-OMAT AR film. Radioactivity at the origin was counted and the percent hydrolysis by phospholipase A₂ determined.

As shown in Figure 15, the recombinant human phospholipase A₂ activities is 25 significantly inhibited at 0.1-1.0 μ M concentrations of *N. scutatus* phospholipase A₂ inhibitor. The IC₅₀ of *N. scutatus* phospholipase A₂ inhibitor for recombinant human non-pancreatic phospholipase A₂ is approximately 1.5 μ M.

- 41 -

EXAMPLE 6

pH Optimum and temperature stability of *N.scutatus* venom phospholipase A₂ inhibitor

5 The pH stability was investigated by altering the pH of the solution in which the SPP (0.4mg/mL) was dissolved and then testing this in the phospholipase A₂ assay. The assay was performed as described in Example 2, using *N.scutatus* venom as the phospholipase A₂ source (1/200 dilution of a 1mg/mL with 10pPC as substrate). All samples were performed in triplicate with appropriate positive and negative controls. The pH values 10 tested were: 2, 4, 6, 7, 8, 9, 10 and 12.

The temperature stability was assessed in the same manner as the pH stability. Samples were heated, or cooled, at the appropriate temperature and then immediately tested in the phospholipase A₂ assay. Temperatures examined were: 4°C, 25°C, 37°C, 50°C, 60°C, 15 70°C, 80°C, 90°C and 100°C.

For both experiments samples were not preincubated with the venom as the stability of the phospholipase A₂ under the varying pH and temperature values could not be assured. However, the ratios phospholipase A₂ to inhibitor used in the preceding 20 Examples were maintained in this procedure.

NSI was stable in the pH range 4.0-12.0, with activity declining at extreme acidic pH values. NSI was also stable at the temperatures tested. Thus, NSI is a highly-stable protein.

25

EXAMPLE 7

Activity of the *N.scutatus* phospholipase A₂ inhibitor following de-glycosylation of the α -chain

30 The α -chain was deglycosylated with N-glycosidase F (cleaves N-linked sugars) or O-

glycosidase (cleaves O-linked sugars) as follows: 10 μ g (10 μ L) of the SPP was denatured with an equal volume of 1% (w/v) SDS followed by boiling for 2 minutes. To this 90 μ L 20mM sodium phosphate buffer, pH 7.2, 50mM EDTA, nonidet P-40, 0.5% v/v was added followed by a further 2 minutes boiling. The SPP was then incubated with 0.4U N-5 glycosidase or 2.5mU O-glycosidase for 16 hours at 37°C. A sample was then run on SDS-PAGE under reducing conditions. The gel was then blotted onto nitrocellulose and sugar residues detected with the Boehringer Mannheim DIG glycan detection kit as per manufacturers instructions. Appropriate controls were performed. A duplicate gel was run and silver stained to determine the shift in molecular weight of the α -chain following 10 deglycosylation.

It was determined that only N-linked sugars were present on the α -chain. As such, the α -chain was deglycosylated with N-glycosidase F as outlined above except that SDS and nonidet P-40 were omitted as were the boiling steps. This was to ensure that NSI was not 15 irreversibly denatured by boiling or SDS treatment. Deglycosylation was confirmed with the DIG glycan detection kit and the shift in molecular weight following SDS-PAGE. The sample was then assayed for inhibitory activity on *N.scutatus* venom (1/300 dilution of 1mg/mL solution dissolved in saline/0.1% w/v BSA). Native NSI was used as the positive control.

20

The formation of the NSI intact complex following deglycosylation of the α -chain was determined using size exclusion chromatography. The deglycosylated SPP (containing NSI) was run on a Superdex 75 column (3.2mm x 30mm) using the Pharmacia SMART HPLC system in 0.1M NH₄OAc pH 7.0. The column was calibrated with 25 molecular weight standards. Native SPP was run as a positive control.

The de-glycosylated NSI retained activity compared to the native inhibitor, consistent with observations in respect of both *A.bilineatus* and bee venom phospholipase A₂ inhibitors.

30 However, the de-glycosylated NSI exhibited a different elution profile from Superdex 75

- 43 -

compared to the native inhibitor, with significantly higher molecular weight species being present, possible due to the formation of functional high molecular weight aggregates involving the de-glycosylated α -chain. Additionally, the size of the assembled NSI complex differed slightly from native NSI due to the altered glycosylation status of the 5 assembled complex.

EXAMPLE 8

Determination of the *N. scutatus* phospholipase A₂ inhibitor complex formation with notexin

10

The native molecular weight of NSI was determined using size exclusion chromatography using a Pharmacia Superose 12 HR10/30 column attached to a Waters 600 series HPLC system. Elution buffer was 0.1M NH₄OAc, pH 7.0 at a flow rate of 0.5mL/min. NSI (60 μ g)

15 was loaded on the column. The column was calibrated with molecular weight standards.

The formulation of a stable complex between NSI and notexin was also investigated using size exclusion chromatography. The SPP (150 μ g) and notexin (100 μ g) were incubated for 30 minutes followed by elution on the Superose column. The NSI and notexin mixture eluted from Superose 12 immediately before NSI, confirming the ability of NSI to bind to

20 notexin.

The peaks were collected and components identified by SDS-PAGE followed by silver staining to confirm their identities.

25

EXAMPLE 9

Effects of NSI on Cancer Cells *in vivo*

Nude mice experiments were conducted to investigate the effects of NSI (a PLA₂ inhibitor), NS398 (a COX2 inhibitor) and a combination of NSI and NS398 on the growth 30 of cancers in the mice. A total of 8 cell lines were employed. The cancers selected were

- 44 -

PC-2 [ATCC No. CRL1435] which is a prostate cancer cell line (adenocarcinoma epithelial cells) and LNCaP [ATCC No. CRL10995/ CRL1740] which is also a prostate cancer (carcinoma epithelial cells). In addition, the following cell lines were also used.

5	Tca 8113	tongue cancer
	Acc-2	adenoid cystic keratin
	Acc-3	adenoid cystic carcinoma
	BGC-823	stomach carcinoma (62 yrs male) [epithelial like cell]
10	SGC-7901	stomach adenocarcinoma (56 yrs female) [metastasis to lymph node, epithelial like cell]
	SPC-A-1	lung adenocarcinoma

NSI, NS398 or the combination of NSI and NS398 were administered by either
15 subcutaneously or intraperitoneally. After the first injection, administration was 3 times a
week for 6 weeks.

Two tumours were induced per animal as follows:

20 1st phase: 1 x 8 cell lines 8 mice

2nd phase:

Per mode of injection:

25	NSI	7 mice
	NS398	7 mice
	NSI/NS398	7 mice
	Control	<u>7 mice</u>

(2 modes of injections;
30 subcutaneous and intraperitoneal) 56 mice

- 45 -

Total 64 mice

Number of cells required:

5 1st phase:

8 tumour cells lines 10^6 cell/line

2nd phase:

2 tumours 28×10^6 cells/turnour

10 Dosages of the compounds were as follows:

NSI (MW 110,000): $1.0 \mu\text{mol}/\text{kg}$ body weight

Approximately 2.2 mg of NSI was administered per mouse over 3 injections.

NS398 (MW314): $0.3-5 \mu\text{g}/\text{kg}$ body weight.

15

Approximately 31.4 μg of NS398 was administered per mouse over 3 injections.

The following time line for the nude mice experiments was observed:

20 1st Phase:

	<u>Week</u>
Grow cells (8 cell lines) to 10^6 cells	1st-2nd

2nd Phase:

Inoculation to induce tumours (8 cell lines)	3rd-6th
--	---------

Grow cells (2 cell lines) 28×10^6 cells	4th-6th
--	---------

25 3rd Phase:

Animal inoculations and tumour growth	6th-12th
---------------------------------------	----------

Total:

12 weeks

The results are shown graphically in Figures 2 to 7 and in corresponding Tables 3 to 8,

30 respectively.

- 46 -

Referring to the figures and the tables, the cancer cell line tested was BGC823. Day 0 is the day the first dose of inhibitor is administered. The volume of cancer is then determined. As shown in Figure 2 (Table 3), cancer size is reduced in the presence of NSI inhibitor (subcutaneously administered). In Figure 3 (Table 4), the inhibition by NSI is even greater when intraperitoneally administered.

Figure 4 (Table 5) and Figure 5 (Table 6) show the effects of NS398 administered by the subcutaneous and interperitoneal routes, respectively. Again, inhibition of the cancer is observed. The combination of NSI and NS398 is shown in Figure 6 (Table 7) and 7 (Table 10 8).

These data show that inhibiting COX2 does reduce the volume of cancer in nude mice.

- 47 -

EXAMPLE 10

Effects of NSI on Cancers *in vivo*

The methodology of Example 5 was again applied to nude mice using the same protocol.

5

The results are shown in Figures 8 to 12.

Figure 8 shows the growth of tumours BGC-823 and SGC-7901 in nude mice without inhibitors.

10

Figures 9 and 10 show the effects of tumour growth in the presence of NSI or NS398 (Figure 9) or the combination of NSI and NS398 (Figure 10).

Similar data are shown in Figures 11 and 12.

15

Again NSI and NS398 individually inhibit tumour growth but the combination of NSI and NS398 is not demonstrably better than the individual inhibitors.

In summary, the data indicate that NSI is a potent inhibitor of BCG823 abdominal growth
20 and is more effective than NS398. Synergism between NSI and NS398 has not yet been observed.

EXAMPLE 11

Effects of cytokines on Inhibition of Cancer by NSI

25

sPLA₂ expression is enhanced by cytokines such as IL-1 TNF α . Monoclonal antibodies are commercially available against these cytokines as well as PLA₂ and COX2. Those antibodies are used to monitor sPLA₂ expression and/or activity in response to PLA₂ inhibitors and in response to inhibitors of the cytokines. By reducing PLA₂ expression or 30 activity, tumour growth is expected to be greatly reduced.

- 48 -

EXAMPLE 12

Effects of NAI on cancers *in vivo*

Similar results to these described above are obtainable using NAI from *N. ater*.

5

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in 10 this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

- 49 -

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TABLE 3

BGC-823 Control Epidermis

NSI Epidermis

Day	control	max	min	inhibitor	max	min	t	P
11	154.76	190.20	119.32	60.17	79.66	40.68	2.34	0.05
14	496.89	615.26	378.52	214.69	323.91	105.47	1.75	>0.1
16	741.78	852.48	631.08	377.68	555.34	200.02	1.74	>0.1
18	1097.65	1260.81	934.49	567.41	835.72	299.10	1.69	>0.1
21	1680.40	2072.09	1288.71	910.51	1320.88	500.14	1.36	>0.1
23	2341.97	2753.51	1930.43	1216.20	1761.93	670.47	1.65	>0.1
25	2786.51	3228.10	2344.92	1623.79	2327.66	919.92	1.40	>0.1

15

TABLE 4

BGC-823 Control Abdomen

NSI Abdomen

Day	control	max	min	inhibitor	max	min	t	P
11	228.10	305.27	150.93	10.24	18.04	2.44	2.81	0.02
14	640.70	852.78	428.62	5.00	8.73	1.27	3.00	0.02
16	920.72	1194.55	646.89	10.39	19.09	1.69	3.32	0.01
18	1430.13	1833.83	1026.43	46.77	85.94	7.60	3.41	0.01
21	2046.44	2608.89	1483.99	119.95	209.96	29.94	3.38	0.01
23	2461.80	3134.96	1788.64	168.25	284.63	51.87	3.36	0.01
25	3136.62	4004.11	2269.13	303.86	508.56	99.16	3.18	0.01

- 50 -

TABLE 5

BGC-823 Control Epidermis

NS398 Epidermis

Day	control	max	min	inhibitor	max	min	t	P
11	154.76	190.20	119.32	14.61	25.33	3.89	3.78	0.01
14	496.89	615.26	378.52	49.56	89.87	9.25	3.58	0.01
16	741.78	852.48	631.08	43.70	73.69	13.71	6.09	0.01
18	1097.65	1260.81	934.49	108.62	187.65	29.59	5.46	0.01
21	1680.40	2072.09	1288.71	206.84	341.27	72.41	3.56	0.01
23	2341.97	2753.51	1930.43	325.39	535.64	115.14	4.36	0.01
25	2786.51	3228.10	2344.92	513.48	837.84	189.12	4.15	0.01

15

TABLE 6

BGC-823 Control Abdomen

NS398 Abdomen

Day	control	max	min	inhibitor	max	min	t	P
11	228.10	305.27	150.93	18.29	27.67	8.90	2.70	0.02
14	640.70	852.78	428.62	73.22	106.50	39.94	2.64	0.05
16	920.72	1194.55	646.89	146.69	207.98	85.39	2.76	0.05
18	1430.13	1833.83	1026.43	246.95	347.59	146.31	2.84	0.02
21	2046.44	2608.89	1483.99	445.02	615.12	274.91	2.73	0.02
23	2461.80	3134.96	1788.64	632.81	865.08	400.53	2.57	0.05
25	3136.62	4004.11	2269.13	760.08	1024.53	495.62	2.62	0.05

- 51 -

TABLE 7

BGC-823 Control Epidermis

NSI+NS398 Epidermis

Day	control	max	min	inhibitor	max	min	t	P
11	154.76	190.20	119.32	19.49	38.97	0.00	3.34	0.02
14	496.89	615.26	378.52	69.37	138.74	0.00	3.12	0.01
16	741.78	852.48	631.08	112.41	218.77	6.05	4.10	0.01
18	1097.65	1260.81	934.49	187.70	344.66	30.75	4.02	0.01
21	1680.40	2072.09	1288.71	511.63	956.80	66.46	1.97	0.10
23	2341.97	2753.51	1930.43	575.60	1068.35	82.86	2.75	0.05
25	2786.51	3228.10	2344.92	745.02	1367.89	122.14	2.67	0.05

15

TABLE 8

BGC-823 Control Abdomen

NSI+NS398 Abdomen

Day	control	max	min	inhibitor	max	min	t	P
11	228.10	305.27	150.93	58.66	89.99	27.33	2.03	0.10
14	640.70	852.78	428.62	131.83	215.02	48.63	2.23	0.05
16	920.72	1194.55	646.89	171.00	279.20	62.79	2.55	0.05
18	1430.13	1833.83	1026.43	282.63	449.52	115.74	2.63	0.05
21	2046.44	2608.89	1483.99	689.83	1142.44	237.22	1.88	0.10
23	2461.80	3134.96	1788.64	897.55	1478.49	316.61	1.76	>0.1
25	3136.62	4004.11	2269.13	1104.17	1752.86	455.48	1.88	0.10

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